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Promotion of Seed Germination by Cyanide

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ABSTRACT

Potassium cyanide at 3 μM to 10 mM promotes germination of *Amaranthus albus*, *Lactuca sativa*, and *Lepidium virginicum* seeds. L-Cysteine hydrogen sulfide lyase, which catalyzes the reaction of HCN with L-cysteine to form β-L cyanoalanine, is active in the seeds. β-L-Cyanoalanine is the most effective of the 23 α-amino acids tested for promoting germination of *A. albus* seeds. Aspartate, which is produced by enzymatic hydrolysis of asparagine formed by hydrolysis from β-cyanoalanine, is the second most effective of the 23 amino acids. Uptake of aspartate-4-^{14}C is much lower than of cyanide.

Radioactive tracer in K^{14}CN shows uptake of about 1.5 μmoles of HCN per gram of *A. albus* and *L. sativa* seeds after 20 hours of imbibition. Extracts of the seeds gave high ^{14}C activity in β-cyanoalanine, asparagine, and aspartate. The acid-hydrolyzed protein extract gave high activity only in aspartate. Tests were negative for free cyanide in the seed. Respiration of the seed is inhibited more than 75% by KCN and by KN_3 at 10 mM. Azide at greater than 1.0 mM inhibits the promotion of germination by cyanides. Neither 0.1 mM KCN nor KN_3 inhibit O_2 consumption, whereas lower concentrations promote germination. It is concluded that the high rate of utilization of cyanide in the reaction to form β-L-cyanoalanine and the subsequent incorporation into protein limit any inhibition of oxygen consumption. The promotion of seed germination is substrate-limited by asparagine-aspartate, which is required for protein synthesis.

Promotion of germination of dormant rice and barley seeds by cyanides has been observed by Roberts (12). We have found that seeds of pigweed (*Amaranthus albus* L.) and lettuce (*Lactuca sativa* L. var. Grand Rapids) are also responsive (8). Roberts suggested, and we concurred (8), that the promotion depends on a cyanide-insensitive hydrogen acceptor as an alternative to oxygen. This possibility, however, becomes very doubtful on the basis of evidence presented here. The important, and probably the only, factor for the several kinds of seeds used by us, is the assimilation of cyanides to form L-asparagine, which undergoes some hydrolysis, with possible incorporation of both amino acids into protein.

Our attention was drawn to possible assimilation of cyanides in germinating seeds by E. Conn, who, with his coworkers (1, 3), has observed formation of asparagine in a number of seedlings on cyanide substrates. The cyanide reacts as HCN with L-cysteine, with a K*m* of 0.55 mM, and with O-acetyl serine to form β-L-cyanoalanine, which adds water under enzymatic control to form L-asparagine. The enzymes involved have been partly purified (9). Fowden and Bell (7) found a similar system in *Chlorella pyrenoidosa*, and one having a K*m* near 13 μM was found for formation of β-cyanoalanine from serine in *Escherichia coli* (4). We have identified the reaction with cysteine in imbibed ungerminated seeds and have related it to germination control by protein formed from the incorporated cyanide.

MATERIALS AND METHODS

Seeds and Germination Tests. *A. albus* and *Lepidium virginicum* L. seeds were collected over a number of years in the neighborhood of Beltsville, Maryland and held at −15 C before use. Seeds in germination tests were placed in lots of 100 in duplicate on two filter papers moistened with water or the appropriate solution and held for 2 to 4 days at constant temperature in H_2O-saturated atmosphere. All tests were repeated two or more times. Conditions for the three seed kinds were: *L. sativa* (from commerce), 25 C, dark; *A. albus*, 25 or 30 C, dark, or after 18 hr of continuous far red radiation greater than 690 nm; and *L. virginicum*, 20 C dark with exposure to 22 p-einstein of 600 to 680 nm radiation after 24 hr of darkness. Emergence of the radicle was taken as evidence of germination.

Oxygen Consumption. Seeds were imbibed under nongermination conditions, which were: *L. sativa*, 33 C, dark; *A. albus*, 30 C, continuous far red radiation. *A. albus* seeds were also used under germinating conditions after 18 hr at 30 C in darkness. Oxygen consumption was measured with a Clark polarization electrode at 30 C with 0.3 g (940 seeds) *A. albus* or 100 (0.12 g) *L. sativa* seeds, both in the presence and absence of antibiotics. Initial rates were recorded.

L-Cysteine Hydrogen Sulfide Lyase (adding HCN) Assay (1). Seeds (0.300 g of *A. albus*, 200 *L. sativa*) imbibed under indicated conditions were ground with a Ten Broeck unit, with or without Al_2O_3, at ice bucket temperatures in succession with 2, 1, and 1 ml of 0.1 M tris buffer at pH 8.5, centrifuged 10 min at 12,000*g*, and decanted. Two 0.1-ml aliquots of the combined solutions were removed for protein analysis following the method of Lowry *et al.* (10), standardized against lyophilized bovine serum albumin. *A. albus* seeds, analyzed by microkjeldahl, contained 2.25% nitrogen (14.1% protein). Solutions were brought to room temperature (28 C), and 1.0 ml of 75 mM KCN and 1.0 ml of 15 mM L-cysteine solutions were added to a total volume of 6 ml. After 30 min, H_2S produced was assayed under closure by adding 0.5 ml of 0.09 M $FeCl_3$ in 3.6 N HCl and 1.5 ml of 0.02 M N,N,dimethyl-*p*-phenylene diamine salt in 7.2 N HCl. After 10 min the solution was centrifuged to remove precipitated protein and decanted through a microfilter. The absorbance of the methylene blue formed was read at 650 nm against a blank of minus cyanide. The procedure was standardized against Na_2S. Controls were run without cyanide or cysteine.

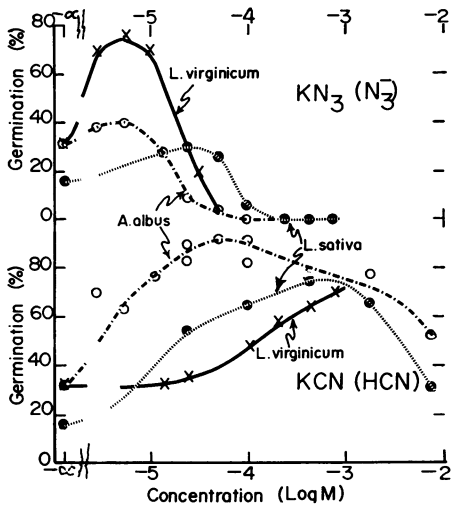

FIG. 1. The per cent germination of *Amaranthus albus, Lactuca sativa,* and *Lepidium virginicum* seeds at 30, 25, and 20 C, respectively, with various initial concentrations of KCN and KN_3 in darkness, except for *L. virginicum* which received 22 p einsteins of 600 to 680 nm radiation after 24 hr imbibition. Germinations of the controls without substrate are shown at zero molarity, or $-\alpha$ in terms of log M.

Methylene blue formation was not followed for lettuce seed because of the brownish coloration of final solutions. Removal of seed coats after short periods of imbibition reduced coloration, but with absorbance values near those of the controls, results were not reliable. Qualitative tests for H_2S were made by bringing the solution under question to 6 N in HCL, and with a moistened lead acetate filter-paper strip under enclosure, noting PbS formation. This was more sensitive than methylene blue formation and was more reliable in detecting low activities of the lyase in seeds.

Uptake of ^{14}C Cyanide and Aspartate-4-^{14}C. One to 1.5 ml solutions of 1.0 mM $K^{14}CN$ (10 μc/mole) or 10, 1.0, or 0.1 mM aspartate-4-^{14}C were used on 0.300 g of *A. albus* or 0.36 g of *L. sativa* seeds on a single filter paper in 4.8-cm Petri dishes under conditions indicated in the tables. At the completion of a run, aliquots of the solutions used were taken and evaporated to dryness at less than 100 C for ^{14}C measurement on a proportional counter. The seeds were then washed with a large volume of water, and a counted number of them were taken for measurement of activity, uncorrected for β-ray absorbance. The washed seeds were ground, as for measurement of the sulfide lyase activity, in 10 mM tris buffer (pH 8.3) and centrifuged at 12,000*g*. The supernatant after decanting was counted directly on an aliquot evaporated to dryness. The remainder was brought to 5% in trichloroacetic acid and held at 2 C for 30 min. After centrifuging and washing, the pellet was heated to 96 C for 15 min in 5% trichloroacetic acid for hydrolysis of RNA. The activity of the solution was measured after centrifuging, evaporating to dryness, and taking up in water. The residual protein activity was measured on an aliquot of a water suspension of the pellet. The protein was hydrolyzed with 6 N HCl for 4 hr at 110 C. The solution was filtered, evaporated to dryness over NaOH, and taken up in water, with activity measurement on an evaporated aliquot.

Other 0.300-g lots of *A. albus* seed were ground in succession with 3, 3, 2, and 2 ml of 50% ethyl alcohol at room temperature. They were centrifuged, and the combined supernatants were evaporated to dryness over $CaCl_2$. The residues after solution in water were used for ^{14}C activity measurement and amino acid separations. Cyanide was measured by difference of activities between extracts evaporated to dryness and after making 1 N in HCl.

Chromatography. The protein hydrolysate and the alcohol extract were resolved on a column of the quaternary ammonium Ag 1.10× exchange resin prepared half in the free base form and eluted, following Roberts *et al.* (13).

The column acid eluates, and the 50% ethanol extracts were chromatographed in one dimension on Whatman No. 1 paper or silica gel plates with solvent A, methanol-pyridine-water (40:2:10, v/v), which resolved glutamic and aspartic acids, asparagine, and β-cyanoalanine, or solvent B, butanone-methyl ethyl ketone-formic acid-water (40:30:15:15, v/v) for resolution of asparagine and aspartic acids. After development with ninhydrin, reaction, as well as non-color-producing zones, were counted directly. They were then eluted with 1 N HCl, evaporated to dryness, and counted.

RESULTS

Seed Germination. Results are shown in Figure 1 for the three species of seeds in 3 μM to 10 mM KCN or KN_3. Maximum promotion of germination was in the region of 0.01 mM for KN_3 and at 0.1 to 1.0 mM for KCN. Germination was reduced to zero by a concentration greater than 0.3 mM KN_3. Inhibition by azide was dominant in the presence of KCN. Thus 0.32 mM KN_3 in the presence of 0.01, 0.1, or 1.0 mM KCN prevented germination for *A. albus* seeds in darkness at 30 C, whereas the value with 0.2 mM KCN alone was 94%. Seedlings on 0.1 to 1.0 mM KCN were larger than controls on water.

Some kinds of seeds show reduced germination in the presence of substrate that provides more than a covering liquid film (5). With *A. albus* seeds, no reduction was shown under 3.0 mm H_2O, and values were reduced by about 50% under 12.5 mm H_2O. The germination decreased more rapidly with depth under 0.5 mM KCN than with water.

Aspartate, asparagine, and cysteine at 0.3 to 10 mM and pH 5 gave less than 2% germination, as did water, with *A. albus* seed given 18 hr far red irradiation at 30 C and then left in darkness at 30 C for 3 days. Under the same conditions, KCN at 10 mM and pH 11 gave 32% germination, which was not changed in the presence of 0.3 to 10 mM cysteine. Measurements of *A. albus* seed in darkness in the presence of aspartate or β-cyanoalanine at various concentrations and pH values are shown in Table I.

Germination tests in darkness of *A. albus* seeds with 21 of the common α-L-amino acids, glycine, and casein hydrolysate at 10 mM and pH 3.5 showed marked enhancement of germination only for glutamic and aspartic acids, which gave greater than 1.52 and 1.25 times control germination (49%), respectively. Enhancements by a factor of 1.16, which are near the

Table I. *Germination of A. albus Seeds in the Presence of L-Aspartate or β-L-Cyanoalanine at 30 C in Darkness*

Concn	pH	Germination with	
		Aspartate	β-Cyanoalanine
mM		%	
10.0	3.5	62	82
10.0	4.2	49	87
10.0	7.0	34	80
1.0	3.5	—	63
0.1	3.5	—	37
0.0	7.2	31	21

limits for significance, were shown for glutamine, glycine, and alanine. Values for other amino acids tested did not differ essentially from those for the water controls. All seedlings appearing at pH 3.5 were injured because of the low pH, as evidenced by stubbing of the radicles, except those of glutamic acid. The low-pH solutions were used to facilitate uptake of the amino acids. Cystine could not be used because of limited solubility, and L-tyrosine was tested at 2 mM.

Oxygen Consumption. Some results are shown in Figures 2 and 3 for oxygen consumption of *A. albus* and *L. sativa* seeds on several concentrations of KCN or KN_3. Such results depend on the rate of entry of the compound used, its possible internal utilization, its effectiveness at the site of action, and whether the seeds are in the course of germinating. Conditions used for results shown in Figures 2 and 3 were such as to suppress germination, being at too high a temperature for *L. sativa,* and under continuous far red radiation for *A. albus.* Under these conditions, 10.0 mM KCN reduces O_2 consumption for *A. albus* seeds to 20% of that of H_2O controls. Azide at 10.0 mM completely suppresses oxygen consumption of *L. sativa.*

Oxygen consumption of the seeds increases greatly as germination proceeds. After 18-hr imbibition in darkness, which is before external sign of germination are evident, 1.0 mM KCN reduces O_2 consumption by *A. albus* seeds to about 50% of that under water. Cyanide at 10.0 mM reduces the consumption to 20%. Azide is less effective than cyanide under these conditions. Oxygen consumption of germinating *A. albus* seeds after 66 hr of water at 30 C in darkness, by which time the shoot was about 1.5 cm long, was 705×10^{-8} l/g seed·min. This was inhibited to 46×10^{-8} l/g seed·min, or by 93% after 10 min in the presence of 3 mM KCN.

Activities of L-Cysteine Hydrogen Sulfide Lyase. Activity

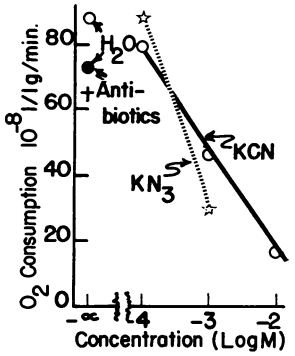

FIG. 2. Oxygen consumption of *A. albus* seeds after 66 hr imbibition under continuous far red radiation in KCN and KN_3 solutions of various concentrations at 30 C.

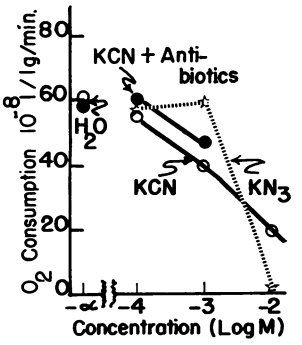

FIG. 3. Oxygen consumption of *L. sativa* var. Grand Rapids seeds after 22 hr imbibition in KCN and KN_3 solutions of various concentrations.

Table II. *Activity of L-Cysteine Hydrogen Sulfide Lyase Extracted from 0.3 g of A. albus Seeds*

Solutions used contained 28 mg of extracted protein.

Imbibition	Germination Conditions	Germination State	Activity[1]	PbS Formation
hr			%	
18	Dark, H_2O	0	113	Medium weak
18	Dark, solution[2]	0	105	
18	FR,[3] H_2O	0	116	
18	FR, solution	0	109	Medium weak
28	Dark, H_2O	Low	120	Medium
28	Dark, solution	Low	133	Strong
36	Dark, H_2O	High	138	Strong
36	Dark, solution	Complete	250	Intense
48	Dark, water	High	200	Intense
48	Dark, solution	Complete	680	Intense

[1] Activity is expressed as *A* at 650 nm given by sample A for water control times 100. Control equivalent to 1.5×10^{-7} moles H_2S/30 min at 28 C.

[2] Solution, 1.0 mM KCN and cysteine.

[3] FR is radiation of >700 nm.

of the lyase extracted for *A. albus* seeds was observable after 18-hr imbibition under far red radiation which prevented germination (Table II). The activity increased with time of imbibition as germination proceeded on water in darkness. It was enhanced in the presence of 1.0 mM KCN and 1.0 mM cysteine, during the imbibition period, the cyanide component of which enhanced germination. There was no evidence, however, that KCN or cysteine increased the lyase activity in nongerminating *A. albus* seeds, An absorbance at 650 nm for methylene blue formation 30% in excess of the control corresponds to production of 1.0 μM H_2S in 24 hr, with consumption of 1.0 μM HCN and 1.0 μM cyanide by 0.3 g of *A. albus* seeds. Nongerminating *L. sativa* seeds after 24-hr imbibition at 30 C showed medium H_2S evolution by PbS formation upon assay.

Distribution of Activity from ^{14}C Cyanide and L-Aspartate-4-^{14}C in Seed Fractions. Seeds, used at 0.25 g/ml of substrate, depleted 1 mM $K^{14}CN$ by about 50% in an 18- to 20-hr imbibition period. The activity recovered in tris-buffered extract was 50 to 70% of the amount disappearing from solution, while extraction of protein was 70%, indicative both of insoluble material and incomplete tissue breakage. The activity in the extracts was about equally distributed between 5% trichloroacetic acid-soluble and -insoluble fractions, which chiefly represent free amino acids and proteins, respectively. These values have considerable error because of compromise on heating to remove volatiles in order to reduce β-ray absorption. Some loss of HCN also might result from action of carbonic acid produced by seed respiration during a test.

Aspartate uptake at pH 4.3 was very low, being of the order of 10^{-5} of the total amount present in the seed, based on an assumed aspartate content and less than 10^{-6} of the aspartate supplied at 10 mM. This small activity probably did not arise from microbial contamination as similar results were obtained in the presence of antibiotics. About half of the aspartate activity disappearing from solution was recovered in the seed protein.

The 50% aqueous ethanol extract of *A. albus* seeds on 1.0 mM $K^{14}CN$ contained ^{14}C-labeled β-cyanoalanine, asparagine, and aspartate as principal activities (Table III). The actual regions of these compounds on the chromatograms accounted for about half of the applied activity. These several compounds were also probable contaminants at the origin and in inter-

Table III. *Chromatography of 50% Ethanol Extract from A. albus Seeds*

The seeds were imbibed at 30 C and 18 hr in the dark. The chromatograms were developed on Whatman No. 1 paper with methanol-pyridine-water (40:2:10, v/v) ascending.

R_F	Compound	^{14}C Activity
		100 cpm
Origin	—	25
7	—	5
26	Asparagine	20
42	Aspartate	10
61	β-Cyanoalanine	9
>73		7
Other[1]		15
	Total recovery	91
	Activity used	90

[1] Intermediate regions between R_F 7 and 73.

Table IV. *Column Chromatography of ^{14}C-labeled Protein Hydrolysate from A. albus Seeds*

The seeds were imbibed in 1.0 mM $K^{14}CN$ at 30 C, for 19 hr in the dark. The method of Roberts *et al.* (13) was used for chromatography.

Eluant	Column Volumes	^{14}C Activity
		1000 counts/min·tube
H_2O	3	4.4
0.04 N NH_4 acetate	3	1.5
0.01 N NH_4OH	3	0.7
	6	4.3
0.05 N HCl	2	12.8
1.0 N HCl	1.5	55.0
	Total eluted	78.6
	Activity used	77.0

mediate regions as well because of streaking by heavy loading. Column chromatography of the ethanol extract and of a protein hydrolysate showed 30 and 70%, respectively, of the activity in 1.0 N HCL eluate, which is glutamic and aspartic acids. Chromatography on paper of this fraction gave activity only in the aspartate region, asparagine being hydrolyzed to aspartate. The 0.05 N HCL eluate of the protein hydrolysate (Table IV) also contained aspartate. The order of 10 to 20% of the total activity in the protein hydrolysate might be in other amino acids than aspartic and asparagine, suggestive of some turnover of the last two into the amino acid pool.

Tests for cyanide in the extracts by differences in activities before and after acidifying and evaporating to dryness gave no measured difference, outside of a variation of about 5% of the total count. This test has a high variance because of dependence on the difference between two large count numbers.

DISCUSSION

Conn and Butler (3) and Fowden (6) have reviewed the literature on the several ways in which HCN is assimilated by seedlings, plants, algae, and microorganisms. The pattern found here for nongerminating, noncyanophoric seed follows that worked out by Blumenthal *et al.* (1) as present in the cyanophoric sorghum, flax, and white clover seedlings and the noncyanophoric barley, pea, and red clover seedlings. HCN, present by hydrolysis, is the predominant cyanide species in the KCN solution used as substrates. It reacts with cysteine present in the seeds, as evidenced by the evolution of H_2S and the identification of β-cyanoalanine. When $K^{14}CN$ is used, a moderate percentage of the label appears in L-asparagine and L-aspartate, as well as in the asparagine-aspartate component of protein. These are expected products from β-cyanoalanine in reasonable yields. The system of reactions involved is shown in Figure 4.

Castric *et al.* (2) found that β-cyanoalanine hydrolyase extracted from blue lupine seedlings did not hydrolyze asparagine to aspartate. E. E. Conn indicated to us (private communication) that he had failed to find asparaginase activity in extracts from several species at the 3 to 7-day-old seedling stage of development. In view of these findings, we further examined the 50% ethanol extracts from *A. albus* by column chromatography on the quaternary ammonium resin, which affords a wide separation of asparagine and aspartate. These findings of labeled aspartate are not necessarily in conflict because of the continuous exposure of seeds in our work to the $K^{14}CN$ solutions. A very slowly acting asparaginase would be adequate to account for our results.

The aspartate-asparagine incorporation into protein appears to be the factor enhancing germination of *A. albus, L. sativa,* and *L. virginicum* seeds on cyanide substrates. If the protein or proteins formed contain on the order of 12% asparagine + aspartate, then on the order of 1% new protein in the *A. albus* seed is formed in 1.0 mM KCN in 18 hr, which is near the concentration giving maximum germination (Fig. 1). This would correspond to an activity of the lyase of 130 (sample/control times 100) functioning for 18 hr, which is about the observed amount (Table II). Also about 1% of the cysteine-cystine present, either free or in protein, is utilized. While a particular protein might appear for the first time or be greatly increased, this could be affected by existing RNAs and requisite enzymes (11). Of pertinence here, but not addressed by us, is the question of what protein or proteins might be limiting to germination.

If cyanide acts to form protein or an amino acid which is otherwise a limiting factor in germination, then β-cyanoalanine, asparagine, and aspartate might be effective, provided they are not limited by uptake. Effectiveness is most evident for β-cyanoalanine (Table I). Aspartate was taken up by *A. albus* seeds to only a minor extent under favorable germination conditions. As the acidity is increased below pH 4.2, with resultant injury to the seedling, the percentage of germination increases (Table I). Asparagine had no effect on germination under the conditions tried; it, like aspartate, probably was limited in entry. Thus effectiveness of cyanide in stimulating germination is fully indicated as resulting from protein synthesis through β-cyanoalanine as an intermediate.

The promotive effect on germination of β-cyanoalanine and its immediate products as a reducing of substrate limitation for protein synthesis is in accord with the ineffectiveness of the other common α-L-amino acids, except as they might take part in transamination. That glutamate might be partially effective is expected because of probable activity of a glutamic-

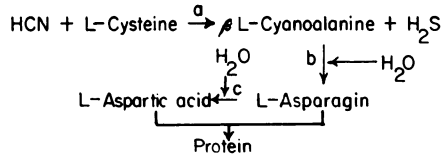

FIG. 4. The reaction scheme for incorporation of carbon from HCN into protein of several kinds of seeds. Enzymatic activities involved are (a) L-cysteine hydrogen sulfide lyase, (b) β-L-cyanoalanine lyase, and (c) and L-asparaginase.

aspartic transaminase (glutamic-oxaloacetic transaminase). It should be borne in mind that effectiveness of uptake for the various amino acids can be limiting, but it is not apt to be very specific.

The results for oxygen consumption by the *A. albus* seeds indicate that any cyanide-insensitive consumption is less than 20% of the cyanide-sensitive value. Germination decrease with increased cyanide concentration above 1.0 mM (Fig. 1), with corresponding decrease in oxygen consumption (Figs. 2 and 3), indicates that normal cyanide, azide-sensitive respiratory system is being inhibited at the higher concentrations of cyanide. This indication is also supported by the increased inhibition of germination of submerged seeds by cyanides contrasted with results with water. The germination results with submerged seed, moreover, indicate that the germination process in *A. albus* seeds does not require a high oxygen supply. This insensitivity to O_2 supply is a quite likely factor enhancing display of a promotive effect of cyanide on germination.The rapid metabolism of cyanide internal to the seed, as shown by its absence upon testing, is favored by the low K*m* for reaction to form β-cyanoalanine. The seeds, in short, are a system poised between rates of uptake and utilization of cyanide. Thus results for oxygen consumption do not support a possible cyanide-insensitive respiration, as we previously assumed (8).

Neither cyanide nor azide measurably influenced oxygen consumption at concentrations of 0.01 mM (Figs. 2 and 3). The promotive effects of azides on germination at these and lower concentrations are as striking as those for cyanide, particularly for *L. virginicum* seeds, which involve an interaction with phytochrome transformation by light. Neither azide nor cyanide causes germination of *L. virginicum* seeds in darkness. We have neither resolved what the azide ion might be doing to effect this promotion nor the nature of the interactions with change of phytochrome.

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